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Genetic Basis of Antibiotic Resistance in Clinical Isolates of *Streptococcus gallolyticus* (*Streptococcus bovis*)

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Among 128 *Streptococcus gallolyticus* (*Streptococcus bovis*) isolates, 77.7% were resistant to tetracyclines and contained *tet*(M) and/or *tet*(L) and/or *tet*(O). A total of 59.4% had macrolide resistance and contained *erm*(B) and, rarely, *mef*(A). Among the one-third of isolates highly resistant to kanamycin and streptomycin, most harbored *aphA3* and *aad-6* genes.

Streptococcus gallolyticus is a member of the *Streptococcus bovis* group, which has recently undergone extensive taxonomic revisions (7, 8, 11). Most isolates belonging to biotypes I and IIa of the *S. bovis* species have been reclassified as *S. gallolyticus*. This species is one of the pathogens most frequently responsible for endocarditis and is presently a leading cause of this infection in certain countries, such as France (3). Moreover, an association between *S. bovis* endocarditis and carcinoma of the colon has been documented, and bacteremia with organisms previously identified as *S. bovis* may be considered as a sentinel of malignancy in the gastrointestinal tract (13). Combination of a penicillin with an aminoglycoside is recommended as a therapy of choice to treat endocarditis due to *S. gallolyticus*. Although penicillin resistance in *S. gallolyticus* has not yet been reported, isolates resistant to erythromycin and related antimicrobials, to tetracyclines, and to high levels of kanamycin have been previously described (12). The aim of the present study was to assess the prevalence of macrolide, tetracycline, and aminoglycoside resistance and to characterize the mechanisms of resistance to these antibiotics in strains of *S. gallolyticus* isolated mostly from blood cultures or cardiac vegetations.

A collection of 135 streptococci identified as *S. bovis* biotype I or II.2 and obtained between 1994 and 2003 from clinical samples was studied. Seventy-two were isolated from blood cultures or cardiac vegetations and were responsible for endocarditis. Of these endocarditis isolates, 46 were from various French hospitals (including 23 kindly provided by J. Etienne and F. Vandenesch), and 26 came from Belgium and The Netherlands, kindly provided by W. H. F. Goessens. Fifty-six other isolates were from blood cultures obtained in French hospitals, four were from other clinical samples, and three were isolated in one cat and two dogs. All strains were stored at −80°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 20% glycerol until being tested.

All isolates were identified as *S. gallolyticus* using the API 20Strep and rapid-identification 32Strep systems (bioMérieux). Species identification was also performed by amplification and sequencing of the *sodA* gene as previously described (7). Among the strains responsible for endocarditis, 71 (98.4%) belonged to the species *S. gallolyticus*, whereas the remaining strain was identified as *Streptococcus lutetiensis.* Furthermore, for the 56 strains isolated from blood cultures, 50 (89.3%), 3 (5.3%), 2 (3.6%), and 1 (1.8%) were identified as *S. gallolyticus*, *Streptococcus pasteurianus*, *Streptococcus infantarius*, and *S. lutetiensis*, respectively. Only the 128 *S. gallolyticus* strains were retained for the study. Screening for antibiotic resistance was first carried out by the disk diffusion method with Mueller-Hinton plates containing 5% sheep blood (Bio-Rad, Marnes-la-Coquette, France) in accordance with the guidelines of the Comité de l'Antibiogramme de la Société Française de Microbiologie (2). The resistance phenotypes of erythromycin-resistant *S. gallolyticus* were further characterized by the agar diffusion method using erythromycin and clindamycin disks. Blunting of the clindamycin inhibition zone proximal to the erythromycin disk indicated an inducible type of macrolide-lincosamide-streptogramin B (MLS_B) resistance. Resistance to both erythromycin and clindamycin indicated MLS_B cross-resistance. Susceptibility to clindamycin with no blunting defined the M phenotype (efflux mechanism).

By the disk diffusion technique, no strain was resistant to penicillin, ampicillin, rifampin, vancomycin, teicoplanin, and high levels of gentamicin. Acquired resistances were detected for macrolides, tetracyclines, streptomycin, and kanamycin. MICs of erythromycin, kanamycin, clindamycin, spiramycin, streptomycin, telithromycin, and tetracyclines were determined by the agar dilution method in accordance with the guidelines of the Comité de l'Antibiogramme de la Société Française de Microbiologie (2). Categorization as nonsusceptible (intermediate or resistant) was done on the basis of the following Comité de l'Antibiogramme de la Société Française de Microbiologie breakpoints: erythromycin, >1 μg/ml; clindamycin, >2 μg/ml; kanamycin and streptomycin, >1,000 μg/ml; telithromycin, >1 μg/ml; tetracycline, >8 μg/ml (2).

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TABLE 1. Phenotypes and genotypes of macrolide resistance in *S. gallolyticus* strains

Erythromycin resistance gene(s)[a]	No. of strains with indicated erythromycin resistance phenotype			Total no. of strains
	MLS_B	Inducible MLS_B	M	
erm(B)	62	11	0	73
mef(A)	0	0	1	1
erm(B) + *mef*(A)	2	0	0	2
Total	64	11	1	76

[a] No amplification was obtained with primers specific for the *erm*(A), *erm*(TR), and *erm*(C) determinants.

Seventy-six isolates (59.4%) were intermediate or resistant to erythromycin. Among these, 64 (84.2%) were cross-resistant to erythromycin and clindamycin (MIC at which 90% of the isolates tested are inhibited [MIC_{90}], >128 μg/ml) whereas 11 (14.5%) were susceptible to clindamycin with blunting, indicating an MLS_B inducible phenotype (erythromycin MIC_{90}, >4 μg/ml; clindamycin MIC_{90}, 0.125 μg/ml) (Table 1). One isolate was intermediate to erythromycin but susceptible to clindamycin (MIC < 0.12 μg/ml) without blunting, displaying an M phenotype. The percentages of strains resistant to tetracyclines and to high levels of kanamycin and streptomycin were 77.7, 34.7, and 37.3, respectively. All strains resistant to high levels of kanamycin and nearly all strains highly resistant to streptomycin were coresistant to erythromycin and tetracyclines. Resistances to erythromycin and tetracyclines were also often combined.

We searched for the presence of antibiotic resistance genes by PCR. Total DNA was extracted by using the Instagene matrix (Bio-Rad), and PCR was performed as previously described (9). The primers used to amplify the gene for the integrase of transposon Tn*916*, int_{Tn}, the genes of resistance to tetracyclines [*tet*(M), *tet*(O), *tet*(S), *tet*(T), *tet*(K), and *tet*(L)], to erythromycin {*erm*(A), *erm*(B), *erm*(TR) [a subset of the *erm*(A) class], *erm*(C), and *mef*(A)}, to kanamycin (*aphA-3′*), and to streptomycin (*aad-6*), and the sizes of the amplicons are listed in Table 2. Amplification of DNA from positive controls with the corresponding primers yielded PCR products of the expected size (data not shown).

In streptococci, two major mechanisms accounting for resistance to MLS antibiotics are recognized. Cross-resistance to all MLS antibiotics is due to methylation of the 23S rRNA by a methyltransferase encoded by an *erm* (erythromycin ribosome methylase) gene (5). Resistance to 14- and 15-membered ring macrolides is mediated by a proton-dependent active drug efflux system encoded by the *mef*(A) (macrolide efflux) gene (5). Among the 76 strains resistant to erythromycin, 73 contained *erm*(B)-like genes, 1 contained a *mef*(A)-like gene, and 2 contained *erm*(B)-like genes combined with *mef*(A)-like genes (Table 1). *erm*(C), *erm*(A), and *erm*(TR) determinants were not detected.

Two known mechanisms of resistance to tetracyclines have been reported in streptococci and enterococci: efflux by proton antiporters [Tet(L) and Tet(K)] and ribosome protection [Tet(M), Tet(O), Tet(S), and Tet(T)]. A total of 77.7% of isolates were resistant to tetracyclines, and *tet*(M) was the most prevalent determinant of resistance to tetracyclines, accounting for 97% of tetracycline-resistant isolates. This determinant is usually highly prevalent in gram-positive bacteria resistant to tetracyclines and is often associated with conjugative elements of the Tn*916* family (1). Consistently, the int_{Tn} gene, encoding the integrase of Tn*916*, was found in all strains harboring *tet*(M)-like genes. These results show that tetracycline resistance in *S. gallolyticus* is due to the acquisition of Tn*916*-related transposons. A *tet*(O)-like determinant was detected alone in one isolate and combined with *tet*(M)-like genes in 13 isolates. *tet*(L)-like determinants were found alone in three isolates and combined with *tet*(M) in five isolates, whereas six isolates contained a combination of *tet*(M)-, *tet*(L)-, and *tet*(O)-like determinants. Combination of tetracycline resistance determinants has been reported previously in various gram-positive bacteria (9, 10). The *tet*(K), *tet*(S), and *tet*(T) determinants were not detected.

All isolates highly resistant to kanamycin contained an *aphA-3*-like gene responsible for phosphorylation of kanamycin and all isolates, but four isolates highly resistant to strep-

TABLE 2. Oligonucleotide primers used in this study

Target gene	Forward and reverse primers (5′-3′)	Size of amplified product (bp)
$sodA_{int}$	CCATACGCATACGATGCTCTTGAACC GAGATAGTAAGCATGTTCCCAAACATC	488
$tet(M)_{Tn916}$	GTGGAGTACTACATTTACGAG GAAGCGGATCACTATCTGAG	350
tet(O)	GCGGAACATTGCATTTGAGGG CTCTATGGACAACCCGACAGAAG	538
tet(S)	CGCTACATTTGCGAGACTCAG GGCTCTCATACTGAATGCCAC	569
tet(T)	CAGTGGGAATATAAGGACACGTC CAAGCCTTCTCTACAGCATC	644
tet(K)	GTAGGATCTGCTGCATTCCC CACTATTACCTATTGTCGC	552
tet(L)	GGATCGATAGTAGCCATGGG GTATCCCACCAATGTAGCCG	516
int_{Tn}	GATGGTATTGATGTTGTAGG GGTCTATATTGACAAGACG	528
erm(B)	GGTAAAGGGCATTTAACGAC CGATATTCTCGATTGACCCA	454
erm(A)	TCAGGAAAAGGACATTTTACC ATACTTTTTGTAGTCCTTCTT	423
erm(C)	TCAAAACATAATATAGATAAA GCTAATATTGTTTAAATCGTCAAT	649
mef(A)	AGTATCATTAATCACTAGTGC TTCTTCTGGTACTAAAAGTGG	328
aphA-3′	GGGGTACCTTTAAATACTGTAG TCTGGATCCTAAAACAATTCATCC	848
aad-6	AGAAGATGTAATAATATAG CTGTAATCACTGTTCCCGCCT	978

tomycin contained an *aad-6*-like gene responsible for adenylylation of streptomycin. The streptomycin resistance in the four PCR-negative strains might be due to a chromosomal mutation, as reported previously for enterococci (6). The results reported in this study are similar to those reported previously in two studies on strains isolated in Taiwan (12) and in humans and animals in Belgium (4).

In conclusion, our results show that tetracycline resistance is widespread in *S. gallolyticus*, due to the presence of *tet*(M) probably borne by conjugative transposons such as Tn*916*, which was detected in 77.7% of the isolates studied. Prevalence of erythromycin resistance was also high, which might be explained by the notion that tetracycline and erythromycin resistance determinants are often colocated on large conjugative transposons (1). These results emphasize the need to monitor the epidemiology and the genetic basis of antibiotic resistance in streptococci.

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